

*GLENN MCGALL*

# Oligonucleotide synthesis

## a practical approach

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*Cover illustration.* The design for the cover was based on Figure 5 from Chapter 1, showing the four common heterocyclic bases and numbering system for the primary structure of DNA.

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## Preface

You might wonder what a c designed primarily for wo witnessed the increasingly c glass funnel full of silica ge DNA synthesis machine, w Oligonucleotide synthesis l biologically orientated labo manual available that adeq oligonucleotide chemistry ir book aims to fill that gap.

In order to reduce to ma dramatic expansion in recer the task of oligonucleotide greater demand than RNA, Here I have chosen just t nucleotide chains: phosphit which involve the now estab methods are widely and succ be thoroughly recommende preparation of protected purification of oligonucle (Chapter 5) and purifi oligonucleotides (Chapter 6 series by adding a brief intr theless from a practical ang

In Chapter 7 the methods reflect the more conventiona often larger quantities of sy describes the important role fragments.

My co-authors in this pro the research groups is highly synthesis and many of the at non-specialists. The manuscr only relatively few alteratio unusual pleasure. The enthu has resulted in the remarka original conception.

## Acknowledgement

The editor would like to th Molecular Biology for their h due to Joan Illsley for inval

### Phosphite-triester Method

isolate and purify the product after each chain extension step.

In solid-phase synthesis, the first deoxyribonucleoside is attached through its 3'-hydroxyl group to an inert insoluble support which also acts as a 3'-blocking device. The derivatised support is contained in a sintered glass funnel and reagents are allowed to react for specific times with the terminal deoxyribonucleoside and are then removed by filtration. The support is thoroughly washed with an appropriate solvent before the next reaction is carried out. In this way excess reagents are simply washed away and only the propagating oligodeoxyribonucleotide chains, anchored to the support, are retained within the reaction vessel.

Specifically, synthesis by the phosphite-triester method proceeds in the following steps. First, the 5'-hydroxyl groups of the deoxyribonucleoside attached to the support are deprotected. Next, they are condensed with excess activated 5'-O-dimethoxytrityl deoxyribonucleoside-3'-phosphoramidite solution. The new 3'-5' internucleotide phosphite-triester linkage is then oxidised to the more stable 3'-5' phosphotriester linkage. Any 5'-hydroxyl groups that failed to condense are capped as acetate esters. The dimethoxytrityl group, attached to the last nucleotide coupled, is removed to provide 5'-hydroxyl groups available for coupling with the next solution of activated deoxyribonucleotide monomer. The cycle is repeated until the oligodeoxyribonucleotide of desired sequence is constructed (*Figure 1*).

At the end of the synthesis the support carries a fully protected oligodeoxyribonucleotide. It is systematically deprotected and released from the support to yield the free oligodeoxyribonucleotide which can be purified by h.p.l.c. or by gel electrophoresis.

The development of this procedure was initiated in 1975 when Letsinger introduced a novel method of rapidly coupling two deoxyribonucleosides using reactive phosphite reagents (1). This new procedure had great potential for both oligoribonucleotide and oligodeoxyribonucleotide synthesis. The method involved the reaction of a suitably protected nucleoside with a bi-functional phosphodichloridite to form a nucleoside-3'-phosphomonochloridite. This is reacted with a protected nucleoside. Mild oxidation generated the more stable 3'-5' internucleotide phosphotriester linkage. The procedure is summarised in *Figure 2*.

By 1981 Matteucci and Caruthers (2) had adapted this chemistry to solid-phase oligodeoxyribonucleotide synthesis using deoxyribonucleoside-3'-phosphomonochloridites ( $X = Cl$ ) or monotetrazolides ( $X = -N=N-$ ) with deoxyribonucleoside-derivatised silica gels as the insoluble support (*Figure 3*).

The reactive deoxyribonucleoside-3'-O-phosphomonochloridites and phosphomonotetrazolides, despite their rapid and efficient coupling potentials, are not ideal reagents for oligodeoxyribonucleotide synthesis. They are exceptionally sensitive to hydrolysis and air oxidation, are difficult to prepare and isolate, require special handling techniques, have limited life times in solution and storage, and often contain appreciable amounts of an inert 3'-3' dinucleoside methox-

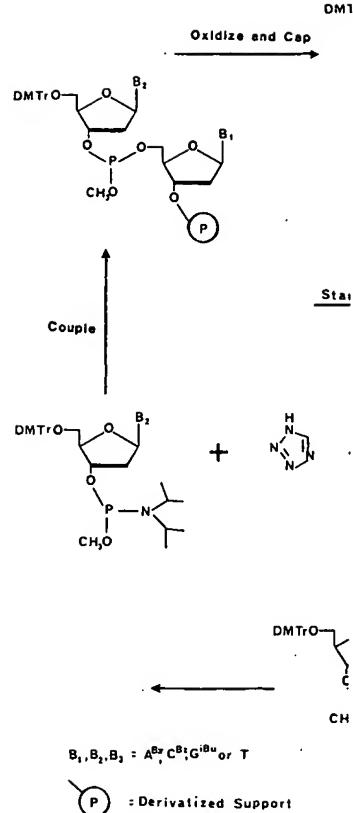


Figure 1. Solid-phase oligodeoxyribonucleotide synthesis.

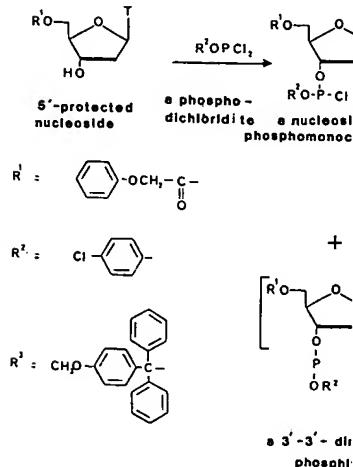


Figure 2. Letsinger's phosphite-triester synthesis.

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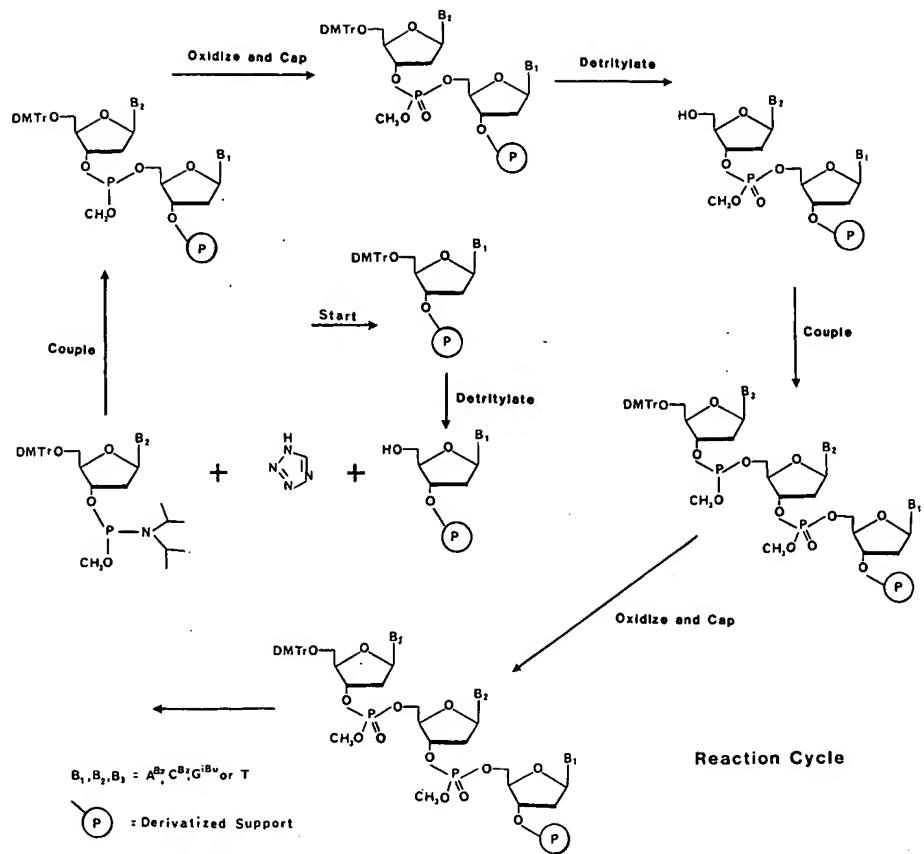


Figure 1. Solid-phase oligodeoxyribonucleotide synthesis by the phosphite-triester method.

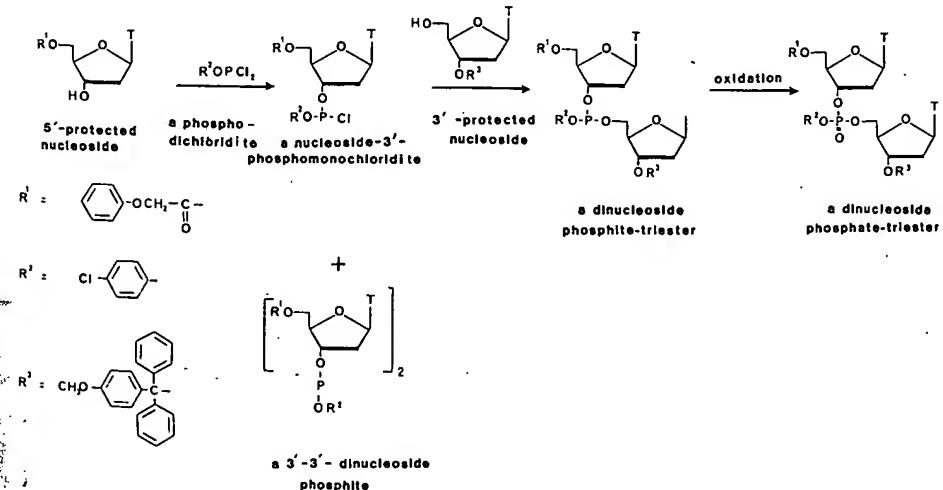


Figure 2. Letsinger's phosphite-triester method.

### Phosphite-triester Method

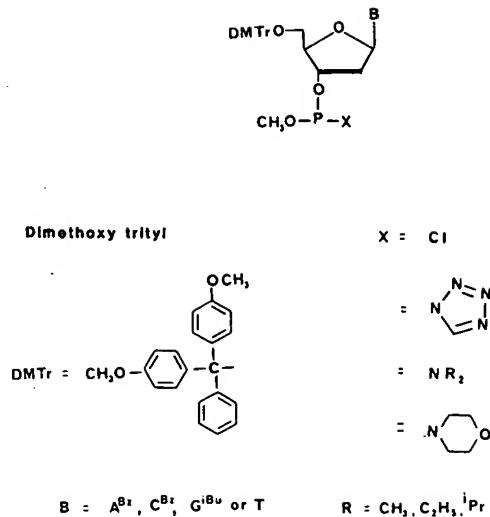


Figure 3. Phosphite reagents introduced by Caruthers *et al.*

yphosphine. These problems were largely resolved when Beaucage and Caruthers (3) introduced a new class of deoxyribonucleoside phosphites: deoxyribonucleoside-3'-O(N,N-dimethylamino)phosphoramidites (Figure 3; X = NMe<sub>2</sub>). These have improved stability and they are not hydrolysed by water or oxidised by air. Consequently they are much easier to prepare and use. Unlike the phosphomonochloridite and phosphomonotetrazolide derivatives, deoxyribonucleoside-3'-O-phosphoramidites cannot react directly with another 5'-hydroxyl-containing deoxyribonucleoside or deoxyribonucleotide. They must first be activated by treatment with a weak acid such as tetrazole.

In the synthesis of deoxyribonucleoside-3'-O(N,N-dimethylamino)phosphoramidites the use of a monofunctional phosphitylating agent, chloro-N,N-dimethylaminomethoxyphosphine ( $\text{MeOPNMe}_2$ ), prevents the formation of any 3'-3'-dinucleoside methoxyphosphine, but excess phosphitylating agent can cause variable amounts of deoxyribonucleoside-3'-O-phosphonate to be produced during work-up of the reaction. The phosphitylating reaction cannot be monitored, or the purity of isolated phosphoramidites estimated using t.l.c. because N,N-dimethylaminophosphoramidites decompose on the silica gel while the chromatogram is developing, even in solvent mixtures containing bases. Hence, the major contaminant in such preparations, deoxyribonucleoside-3'-O-phosphonate, cannot be removed by preparative silica gel chromatography, because of the lability of the phosphoramidites. The phosphonate is an acceptable contaminant because it is inert during oligonucleotide synthesis and its amount, in phosphoramidite preparations, can be estimated by <sup>31</sup>P n.m.r.

Solutions of deoxyribonucleoside-3'-O(N,N-dimethylamino)phosphoramidites in anhydrous acetonitrile display variable stability. In an attempt to discover

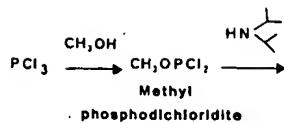


Figure 4. Monomer preparation.

more stable phosphoramidites aminophosphoramidites (Figure 3) that increased steric hindrance McBride and Caruthers (5) cphoramidites (Figure 3; X = deoxyribonucleotide synthesis they are non-hygroscopic, at temperature, and their soluti temperature for at least a wee deoxyribonucleotide synthesis

The commercial availability diisopropyl phosphoramidite ports has made oligodeoxyri Molecular biologists can no defined sequence by manual assembled by sequential addit day and in the USA the cost

Synthesis of deoxyribonucle preparation of phosphitylating group of a suitably protected tions used to prepare the ph should only be performed moisture- and oxygen-sensitiv

Pure chloro-N,N-diisopr available and the preparatio ribonucleoside-3'-O-diisoproj to those familiar with organic synthetic oligonucleotides deoxyribonucleoside-3'-O-ph-

### 3. SYNTHESIS OF PR DIISOPROPYL PHOSPHO

#### 3.1 Preparation of Methyl PI

Methyl phosphodichloridite, 1 and methanol as described by

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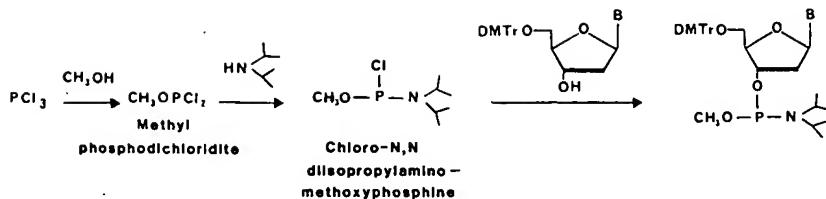


Figure 4. Monomer preparation.

more stable phosphoramidites, Adams *et al.* (4) prepared a series of  $N,N$ -dialkylaminophosphoramidites (Figure 3;  $X = \text{NMe}_2, \text{NEt}_2, \text{NiPr}_2$ ) and demonstrated that increased steric hindrance about the nitrogen atom led to increased stability. McBride and Caruthers (5) confirmed that morpholino and diisopropyl phosphoramidites (Figure 3;  $X = \text{N}(\text{O}), \text{NiPr}_2$ ) are the reagents of choice for oligodeoxyribonucleotide synthesis. Both can be purified easily on a silica gel column, they are non-hygroscopic, and are stable indefinitely as dry powders at room temperature, and their solutions in anhydrous acetonitrile are stable at room temperature for at least a week. They have proved to be ideal reagents for oligodeoxyribonucleotide synthesis.

The commercial availability of pure, stable deoxyribonucleoside-3'-O-diisopropyl phosphoramidites and deoxyribonucleoside-derivatised solid supports has made oligodeoxyribonucleotide synthesis accessible to non-chemists. Molecular biologists can now rapidly synthesise an oligodeoxynucleotide of defined sequence by manual methods using simple equipment. A 20-mer can be assembled by sequential addition of deoxyribonucleotide monomers in less than a day and in the USA the cost per coupling can be less than \$5 for all reagents.

Synthesis of deoxyribonucleoside-3'-O-phosphoramidites involves a two-stage preparation of phosphorylating reagent and then reaction with the 3'-hydroxyl group of a suitably protected deoxyribonucleoside (Figure 4). Warning: the reactions used to prepare the phosphorylating reagent utilise hazardous chemistry and should only be performed by people competent in handling very reactive, moisture- and oxygen-sensitive, pyrophoric agents.

Pure chloro- $N,N$ -diisopropylaminomethoxyphosphine is commercially available and the preparation and purification of 5'-O-dimethoxytrityl deoxyribonucleoside-3'-O-diisopropyl phosphoramidites should present few problems to those familiar with organic synthesis. However, most people interested in using synthetic oligonucleotides will probably prefer to purchase pure deoxyribonucleoside-3'-O-phosphoramidites.

### 3. SYNTHESIS OF PROTECTED DEOXYRIBONUCLEOSIDE-3'-O-DIISOPROPYL PHOSPHORAMIDITES

#### 3.1 Preparation of Methyl Phosphodichloridite

Methyl phosphodichloridite,  $\text{MeOPCl}_2$ , is prepared from phosphorus trichloride and methanol as described by Martin and Pizzolato (6).

## 9. REFERENCES

1. Letsinger, R.L., Finn, J.L., Heavner, G.A. and Lunsford, W.B. (1975) *J. Am. Chem. Soc.*, **97**, 3278.
2. Matteucci, M.D. and Caruthers, M.H. (1981) *J. Am. Chem. Soc.*, **103**, 3185.
3. Beaucage, S.L. and Caruthers, M.H. (1981) *Tetrahedron Lett.*, **22**, 1859.
4. Adams, S.P., Kavka, K.S., Wykes, E.J., Holder, S.B. and Galluppi, G.R. (1983) *J. Am. Chem. Soc.*, **105**, 661.
5. McBride, L.J. and Caruthers, M.H. (1983) *Tetrahedron Lett.*, **24**, 245.
6. Martin, D.R. and Pizzolato, P.J. (1950) *J. Am. Chem. Soc.*, **72**, 4585.
7. Dörper, T. and Winnacker, E.-L. (1983) *Nucleic Acids Res.*, **11**, 2575.
8. Tanaka, T. and Letsinger, R.L. (1982) *Nucleic Acids Res.*, **10**, 3249.
9. Hofle, V.G., Steglich, W. and Vorbruggen, H. (1978) *Angew. Chem.*, **90**, 602.
10. Daub, G.W. and van Tamelen, E.E. (1977) *J. Am. Chem. Soc.*, **99**, 3526.
11. Fisher, E.F. and Caruthers, M.H. (1983) *Nucleic Acids Res.*, **11**, 1589.
12. Froehler, B.C. and Matteucci, M.D. (1983) *Nucleic Acids Res.*, **11**, 8031.

## UPDATE

Recently, a novel form of deoxyribonucleoside-3'-O-phosphoramidite has become commercially available. These are the  $\beta$ -cyanoethyl-N,N-diisopropyl phosphoramidites (13), in which a  $\beta$ -cyanoethyl protecting group replaces the standard methyl protecting group for the internucleotide phosphate. This eliminates the need to use thiophenol for the deprotection of methyl esters at the end of a synthesis.

Instead, oligonucleotides prepared using  $\beta$ -cyanoethyl phosphoramidites can be fully deprotected using only concentrated ammonium hydroxide solution. A 1 hour treatment at room temperature cleaves the succinate linkage and also removes all the phosphate protecting groups by means of a reaction involving a  $\beta$  elimination mechanism. Treatment at 55°C overnight then completes the deprotection by removal of the base protecting groups. In our experience,  $\beta$ -cyanoethyl phosphoramidites display comparable stability, both as dry powders and in anhydrous acetonitrile solution, to that of the corresponding methyl ester-protected phosphoramidites, and they are just as efficient in the internucleotide coupling reactions.  $\beta$ -Cyanoethyl phosphoramidites are now available from American BioNuclear, Biosearch, Biosyntec, Cruachem and Vega Biochemicals.

We have also recently found that the time of reaction for internucleotide couplings can be reduced from 3 minutes to 1 minute without detriment to the synthesis, using either type of phosphoramidite.

Aldrich now supplies anhydrous acetonitrile in 100 ml and 800 ml amounts (catalogue number 27, 100-4) with a water content of less than 0.005%. This acetonitrile can be used directly, without a prior distillation step, to dissolve tetrazole and phosphoramidites. Anhydrous acetonitrile is very hygroscopic. Therefore, a bottle should be opened for the shortest possible time and, after use, all the air inside the bottle should be promptly replaced with argon (using a balloon and needle) before closing and sealing the bottle with Parafilm. A bottle that has been opened and purged with argon more than four times should no longer be considered to be anhydrous. It may therefore be more economical to use 100 ml bottles of anhydrous acetonitrile for oligonucleotide synthesis.

Finally, oligonucleotides can be eluted from C<sub>18</sub> SEP PAK cartridges (see section 6.3.2) using a mixture of acetonitrile (h.p.l.c. grade) and sterile water (1:4).

13. Sinha, N.D., Biernat, J., McManus, J. and Köster, H. (1984) *Nucleic Acids Res.*, **12**, 4539.

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